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The Effect of Calpain-Inhibitor on Muscular Degeneration after Rotator Cuff Tear: A Randomized Clinical Trial in Sheep

MASTERARBEIT

zur Erlangung des akademischen Grades

Master of Medicine (M Med) der Medizinischen Fakultät der Universität Zürich

vorgelegt von

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1. Abstract

Introduction: Tears of the rotator cuff muscle lead to retraction and irreversible degeneration of the muscle, that makes repairs with current methods difficult or to some extent not possible. Different approaches to this matter have been made, for example mechanical re-lengthening or pharmacological treatment. Other studies have investigated the effect of the pharmacological inhibition of the calcium-dependent protease calpain with calpeptin, for preventing muscle atrophy. Yet the role of calpeptin in muscles is unclear. This is what we dedicated our research for.

Material and Methods: computer tomography scans of 18 female, Swiss alpine sheep were analysed after releasing the infraspinatus muscle of the right shoulder. The sheep were divided in two groups, one group (n 12) was administrated calpeptin, the other group (n 6) was administrated an adjuvant and served as a control group. The pathomechanical changes have been studied histologically and with the use of computer tomography during six weeks after muscle release. In the scans and probes were the muscle length, cross sectional area, fatty infiltration and muscle retraction evaluated.

Results: The muscle length decreases over the time and ranges from 16.38 ± 0.17 to 14.87 ± 0.13 cm in the calpeptin (CALP) and 15.51 ± 0.39 to 14.20 ± 0.42 cm in the control group. Significant differences between the two groups can be seen in week 0 and week 4 ($P < 0.05$). The cross sectional area remains mainly constant and ranges from 14.61 ± 0.62 to 13.54 ± 0.62 cm^2 in the CALP and 15.30 ± 0.75 to 13.44 ± 0.79 cm^2 in the control group. The muscle densitiy of the muscle decreases persistently in both groups in the coronal plane as well as in the transverse plane. The retraction of the bone fragment increases constantly over time in both groups and ranges from 0.37 ± 0.03 cm to 1.5 ± 0.19cm in the CALP and 0.70 ± 0.23 to 1.64 ± 0.28 cm in the control group. There is a significant difference between the two groups in week 0 ($P < 0.05\%$).

The contralateral, un-involved side shows constant parameters over the time in both groups.

Conclusion: With calpeptin a positive effect on muscle length and retraction can be shown mainly in the first hour after tendon release. The results show that the muscle retracts quickly probably in association with calpain. Since mechanical factors regulate the muscle mass, there is a possibility of calpeptin, by sustaining the muscle length, preventing degenerative process.

The pathophysiological mechanisms underlying the effects of calpeptin in muscle fibres remain unclear.

2. Abbreviations

CALP	calpeptin
CLAT	contralateral
CSA	cross sectional area
CT	computer tomography
HU	Hounsfield unit
IGF	insulin-like growth factor
OP	operated
ROI	region of interest
DMSO	Dimethylsulfoxid

3. Introduction

Full thickness tears of the rotator cuff can lead to retraction and subsequent irreversible degeneration of the detached muscle; those are the main limiting factors of established repairing procedures [1, 2] of the rotator cuff.

Previous studies have shown, that retraction is mainly caused by shortening of the muscle and then by shortening of the tendon [1]. Once retraction has taken place, it is followed by muscle atrophy and irreversible fatty infiltration [1-3]. These irreversible changes make a repair in an advanced stage of the rotator cuff tear difficult or even to some extent not possible: the muscle needs to be overstretched, leading to damage of fibres and increasing the fatty muscle infiltration [1-3]. Even when repair has succeeded the structural changes of the muscle remain mostly irrecoverable [2, 3]. This issue leaves two approaches: either repair needs to be conducted before retraction has taken place or new treatments procedures are necessary.

The latter has been approached by several researchers with different methods, for example with continuous musculotendinous traction [3], that showed success in re-lengthening the muscle in sheep – but only when fatty infiltration was not advanced beyond 30% (Goutallier stage 1). In that study fatty infiltration could not be reversed, but progression of fatty infiltration could be prevented [3] by muscle re-lengthening. Another approach focuses on pharmacological treatment with anabolic steroids or insulin-like growth factor (IGF) [4] or the combination of both pharmacological and mechanical stimulation [5]. Finally, most of them came to the conclusion, that you can prevent degenerative changes from happening, but you cannot reverse them once they have occurred.

Recent studies investigated the efficiency of the pharmacological inhibition of the calcium-dependent protease calpain with calpeptin, for preventing muscle atrophy [6, 7]. Calpain stands for a family of three proteases that are central to myofibrillar protein degradation through its effect on the proteolysis of the Z-disks that hold sarcomeres in register [8-10]. The critical role of calpain in muscle wasting is emphasized by the recent identification that gene defects in calpain 3 produce muscle dystrophy [11]. Interestingly, calpain also regulates the differentiation of adipocytes in culture [12]. Besides the description of its implication, the (patho-) physiological role of calpain activation in skeletal muscle is not clear [13]. This is where we aim our investigations.

The purpose of this study was to test two different hypotheses: First, that calpain-inhibitor leads to less muscle atrophy and fatty infiltration after tendon detachment, and second, that calpeptin influences muscle length over a reduction of the muscular strength.

4. Material and Methods

4.1. Sheep

An accepted model for chronic rotator cuff injuries is the sheep, due to its similarity of the infraspinatus muscle to the human supraspinatus muscle [14]. The study was carried out with 18 female, white Swiss alpine sheep from the plant Staffelegghof AG, Küttingen Switzerland, not pregnant, at the age of 26.7 (±1.4) months and the weight of 62.8 (±2.5) kg. All sheep were vaccinated against clostridia, pasteurella and foot rot. The infraspinatus muscle was released and the sheep were arranged in two experimental groups. 12 sheep were administered calpeptin (CALP), whereas 6 sheep were administered an adjuvant as a control. (Table 1)

One sheep in the CALP group was excluded due to death after week 2.

4.2. Experiment Protocol and Timetable

As mentioned before the sheep were divided into two groups, one group with CALP administration and another group with adjuvant administration, which served as a control group. Within the groups the right infraspinatus muscle was released and an osmotic pump (Alzet® 2ML4), with the flow rate of 2.5 μl/h giving 12.5 mg/ml substrate in 75% DMSO, was inserted. The contralateral side served as a control. A computer tomography scan and biopsy were taken within an hour after tendon release and after the following two, four and six weeks. The muscle length, cross secitonal area, fatty muscle Infiltration in the coronal and transverse plane and the bone fragment retraction were measured and evaluated. After the killing the entire muscle was removed and histologically investigated. (Figure 1)

Surgeries were performed by the upper extremity team of Balgrist University Hospital (surgeon: PD. Dr. med. Karl Wieser) and a veterinary surgeon at the Vetsuisse Faculty Zurich (surgeon: Dr. med. vet, Karina Klein, DVM-PhD). In all sheep, the infraspinatus tendon at the right shoulder was released by performing osteotomy of the greater tuberosity of the humerus bone. To administer calpeptin locally, the osmotic pumps were inserted with catheters into the muscle at 2.5 cm proximally to the severed infraspinatus tendon. The tube was fastened in place through chinese finger sutures (suture is positioned into the muscle tissue and the ends are wrapped around the tube in a spiral pat-

tern and then tied). To prevent spontaneous healing of the infraspinatus tendon a silicon rubber tube encased the musculotendinous end.

Sheep	All n=18	CALP n=12	control n=6
Age [month]	26.7 ± 1.4	26.7 ± 1.4	26.7 ± 1.4
Weight [kg]	61.8 ± 2.5	64.8 ± 6.6	62.0 ± 2.1

Table 1. 18 swiss alpine sheep divided in two experimental groups. One group received the CALP administration, the other group served as control and received the adjuvant administration.

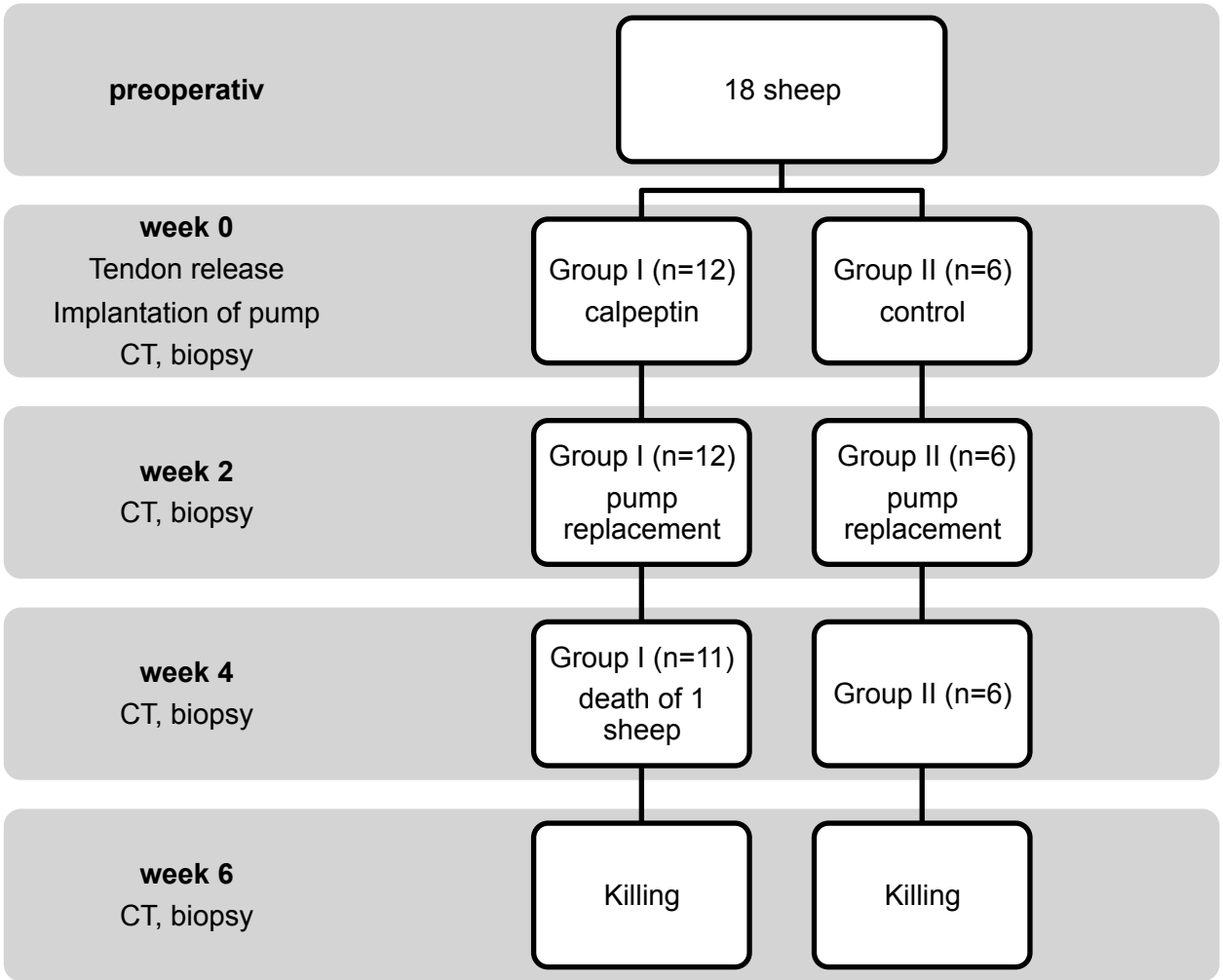

Figure 1. Timetable

4.3. Imaging

Computed Tomography (CT):

A 40-slice CT scanner was used (SOMATOM Sensation Open; Siemens Medical Solutions, Erlangen, Germany). The sheep were scanned between November 2014 and March 2015. All sheep received general anaesthesia for examination in the scanner and were positioned on their back. Image acquisition parameters included an x-ray tube voltage of 120 KVp, a current of 150 mAs and a slice thickness of 1 mm.

4.4. Measurements

The transverse CT images in soft tissue kernel (B20s) were checked for symmetrical scapula position (left and right) and reconstructed in the coronal and sagittal plane in each sheep by a fellowship trained, musculoskeletal radiologist (blinded for review purposes). In case of oblique or asymmetrical scapula position the images were reconstructed as well in the transverse plane in order to get symmetric scapula positions for measurements.

All measurements were performed by a medical student (blinded for review purposes 2015) after a detailed measurement training session, supervised by a fellowship trained musculoskeletal radiologist (blinded for review purposes). The following measurements were performed in all sheep of the CALP-group and the control-group. In every sheep the measurements were done on both sides, on the operated (OP) and the non-involved contralateral side (CLAT) side. (Figure 2)

4.4.1. Muscle Length

The length of the infraspinatus muscle was measured (in cm) in the transverse plane: On the OP side the measurement started from the bone fragment to the point where the scapular spine merges into the scapular body. On the CLAT side the measurement started from tendon insertion at the greater tubercle to the analogue point where scapular spine merges into the scapular body (Figure 3).

4.4.2. Cross Sectional Area

The cross sectional Area (CSA, in cm^2) was measured in the coronal plane (Figure 4): First, the pump was localized in the transverse plane (Figure 5). Afterwards, the coronal

slice lying 1 cm anterior to the anterior pump end was chosen. On that slice the infraspinatus muscle borders were circled for CSA assessment.

4.4.3. Fatty Muscle Infiltration

The density of the infraspinatus muscle was assessed using the Hounsfield units (HU). The HU correlates with the muscle lipid content [15, 16] and is an established form to measure fatty infiltration in CT scans.

Based on HU the muscle was graded for fatty infiltration according to the Goutallier classification, as described previously [17]: stage 0= > 50 HU = < 10% fat, stage 1= 35-49 HU = 11-30% fat, stage 2= 25-34 HU = 30-45% fat, stage 3= 15-25 HU = 45-55% fat, stage 4= <14 HU = > 55% fat.

The density was measured twice, in the coronal and in the transverse plane. The measurements were performed very carefully, excluding adjacent bony borders and air artefacts - in order to avoid false HU measurements.

4.4.3.1. Coronal

The measurements were performed on the same coronal slice as chosen for CSA assessment (Figure 4). The borders of the infraspinatus muscle defined the region of interest (ROI) and the Hounsfield units were assessed.

4.4.3.2. Transverse

Starting with the above chosen coronal image slice on each side a corresponding transverse slice was chosen - running perpendicular to the coronal plane and through the centre of the infraspinatus muscle belly (Figure 6). On this transverse image the borders of the muscle belly defined the borders of the ROI (Figure 7).

4.4.4. Muscle Retraction

On the operated side only, the musculotendinous retraction was measured as the distance of the bone chip to its original insertion side on the humeral head (Figure 8).

4.5. Statistical Analysis

Statistical analysis of the data was performed using the software IBM SPSS Statistics 22 for Mac. The level of significance was set at $P < 0.05$. The data was tested for nor-

mal distribution with the Shapiro-Wilk test, before either parametric or nonparametric tests were performed. The different groups were compared using the unpaired t test along with the assessment of equality of variances by the Levene's test or compared using the Mann-Whitney U test. Missing data were excluded from the analysis. (Table 2)

4.6. Ethics

The study was submitted to the veterinary department of canton Zurich (application number ZH219/2014). The experiment was carried out after their approval and with the most possible ethical husbandry.

Figure 2. CT scans showing the infraspinatus muscle on both sides, the operated and the control side. The pump is situated on the operated right side *(arrow)*, lying in the infraspinatus muscle belly, adjacent to the scapular spine *(asterisk)*, *H* = Humeral head. **(a)** CT of the transverse plane. **(b)** CT of the coronal plane.

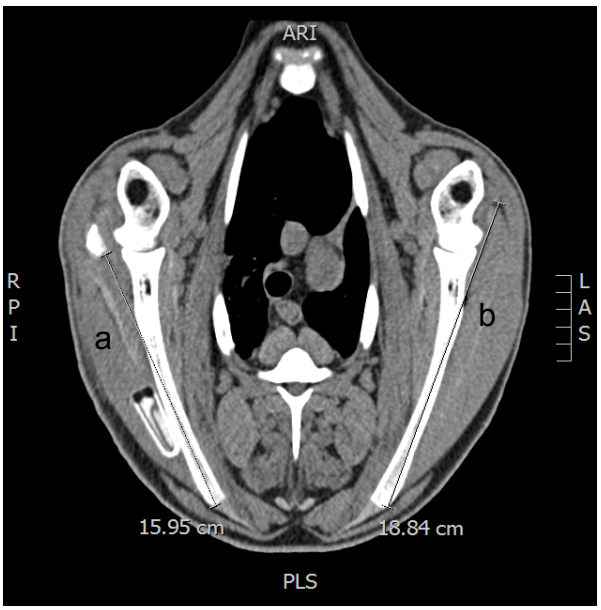

Figure 3. Transverse CT scan showing the measurements of muscle length on both sides: the OP side *(a)* and the CLAT side *(b)*

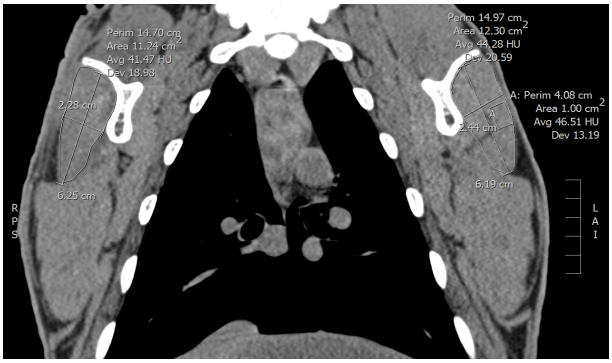

Figure 4. Coronal reconstructed CT image showing measurements of cross sectional area and density on both sides.

Figure 5. Pump in transverse plane and corresponding measurement point for coronal area and density *(dashed line)*.

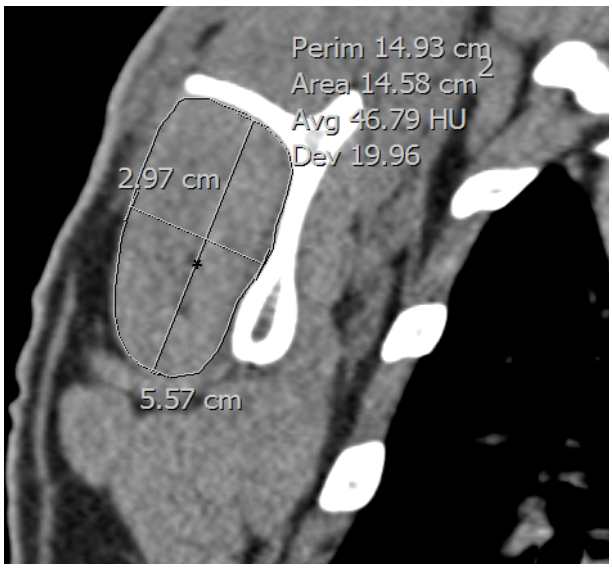

Figure 6. Coronal reconstructed CT image showing the four quadrants in the infraspinatus muscle. The muscle centre *(*)* in the coronal plane served as reference point for the transverse plane in order to conduct density measurements.

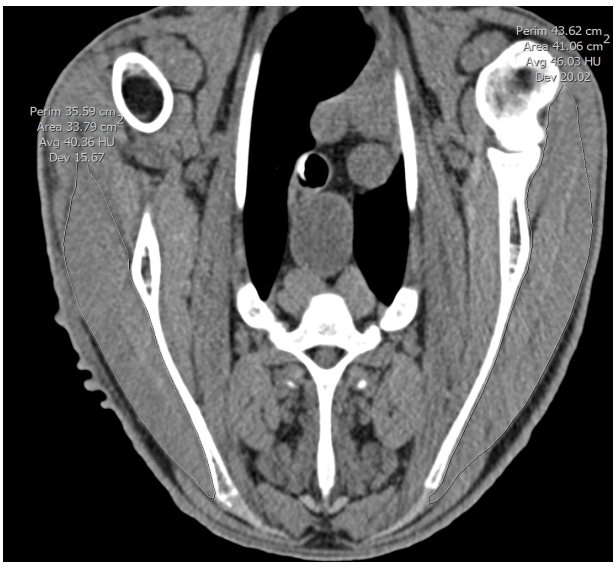

Figure 7. Transverse density measured in transverse plane on both sides for evaluation of the fatty infiltration.

Figure 8. Transverse CT scan showing the measurement of muscle retraction on the operated right side.

5. Results

5.1. Muscle Length

Immediately after tendon release the measurements of the CT show a decrease of the muscle length from its original length on the OP side in both groups (mean ± standard error): The muscle length at this time in the CALP group was 16.38 ± 0.17 cm and in the control group 15.51 ± 0.39 cm. There is a significant difference ($P < 0.05$) between the CALP and the control group. In week 2 the muscle shortens more to a length of 15.57 ± 0.12 cm in the CALP group and 14.87 ± 0.44 cm in the control group. There is no significance difference between the groups. While in week 4 there is a significant ($P < 0.05$) difference between the CALP and the control group. At this point the muscle length amounts to 15.35 ± 0.16 cm in the CALP group and 14.45 ± 0.31 cm in the control group. There is no significant difference between the groups in week 6, where the muscle length decreases to 14.87 ± 0.13 cm in the CALP group and 14.20 ± 0.42 cm in the control group (Figure 9a).

The CLAT, un-involved side shows a constant muscle length over the time in both groups with no significant differences. (Figure 9b)

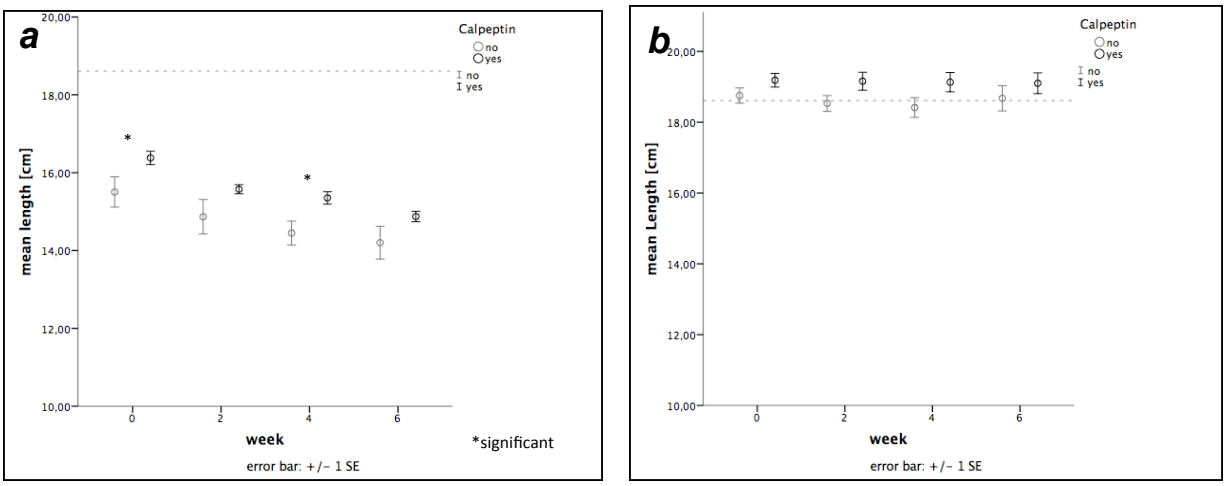

Figure 9. Mean muscle length over time measured in CT. Reference (18.75 ± 0.22) of the original length is the mean muscle length of the control group on the CLAT side, represented here by the grey dashed line. **(a)** OP side: Constantly significant reduction of muscle length from the original length over the six weeks. There are significant differences between the CALP and the control group in week 0 and week 4. **(b)** CLAT side: Constant muscle length with no changes from the reference muscle length.

5.2. Cross Sectional Area

The CSA is quite constant over the time and there are no significant differences between the two groups. In week 0 the CSA was 14.61 ± 0.62 cm^2 in the CALP group and 15.30 ± 0.75 cm^2 in the control group. Then in week 2 the CSA was 15.40 ± 0.52 cm^2 in the CALP group and 14.59 ± 0.88 cm^2 in the control group. In the further process the muscle area was 14.91 ± 0.54 cm^2 in the CALP group and 15.27 ± 1.06 cm^2 in the control group in week 4. In the last CT scans in week 6 the CSA was 13.54 ± 0.62 cm^2 in the CALP group and 13.44 ± 0.79 cm^2 in the control group. (Figure 10a)

On the CLAT side the area remains constant over the six weeks. There are no significant changes, neither from the original area nor between the two groups. (Figure 10b)

5.3. Fatty Muscle Infiltration

5.3.1. Coronal

The coronal muscle density decreases persistently over the six weeks on the OP side. There is an overall reduction from the start value except in week 0 in the CALP group, where the density remains constant. In week 0 the coronal muscle density was 53.91 ± 9.6 HU in the CALP group and 51.39 ± 1.08 HU control group, but there is no significance in the difference between the two intervention groups. In week 2 the density was 46.21 ± 1.06 HU in the CALP group and 49.86 ± 0.93 HU control group. There is a significant difference between the two groups ($P < 0.5$). In week 4 the density was 44.22 ± 1.67 HU in the CALP group while it was 44.32 ± 1.17 HU in the control group. There were no significant differences between the two groups. Then in week 6 the density was 41.63 ± 1.63 HU in the CALP group and 43.04 ± 1.81 HU in the control group, with no significant differences. (Figure 11a)

The CLAT side shows comparatively no significant difference between the two intervention groups. (Figure 11b)

5.3.1. Transverse

In both groups the density in the transverse plane decreases after week 0 over time. Overall there is no significant difference between the CALP and the control group.

Directly after tendon release in week 0 the density was 50.10 ± 0.85 HU in the CALP group and 51.04 ± 1.00 HU in the control group. Then in week 2 the density was 42.25

± 1.30 HU in the CALP group and 46.18 ± 1.03 HU in the control group. In further progress in week 4 the density was 38.43 ± 0.85 HU in the CALP group and 41.47 ± 1.48 HU in the control group. Finally in week 6 the density was 35.45 ± 1.87 HU in the CALP group and 37.06 ± 2.73 HU in the control group. (Figure 12a)

On the CLAT side the density also shows no significant difference between the two groups and remains mainly constant. (Figure 12b)

5.4. Retraction

In week 0 the retraction of the bone fragment is 0.37 ± 0.03 cm in the CALP group and 0.70 ± 0.23 cm in the control group. There is a significant difference between the groups ($P < 0.05$). While in week 2, 4 and 6 there are no significant differences. In week 2 the retraction is 0.86 ± 0.09 cm in the CALP group and 1.01 ± 0.27 cm in the control group. The retraction increases further and in week 4 it is 1.19 ± 0.19 cm in the CALP group and 1.40 ± 0.34 cm in the control group. Finally in week 6 the retraction is 1.50 ± 0.19 cm in the CALP group and 1.64 ± 0.28 cm in the control group. (Figure 13)

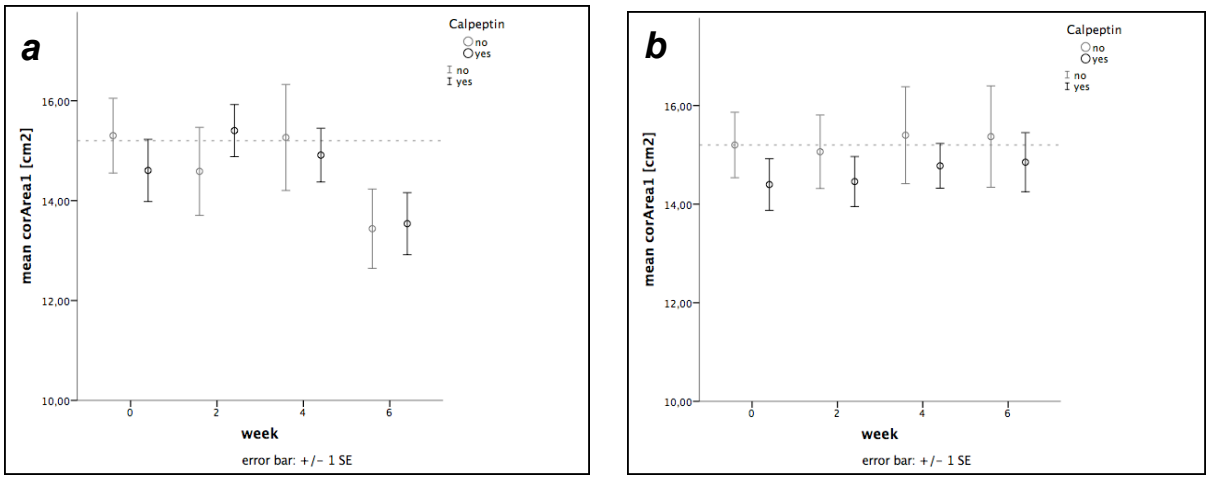

Figure 10. Mean CSA over time measured in CT. As reference (15.20 ± 0.66) of the original area served the mean muscle area of the control group on the CLAT side, represented here by the grey dashed line. **(a)** OPS side: Overall there are no significant differences between the CALP and the control group. Constant area with no change from reference area except in week 6 in CALP group. **(b)** CLAT side: Constant area with no change from reference area. Overall there are no significant differences between the CALP and the control group.

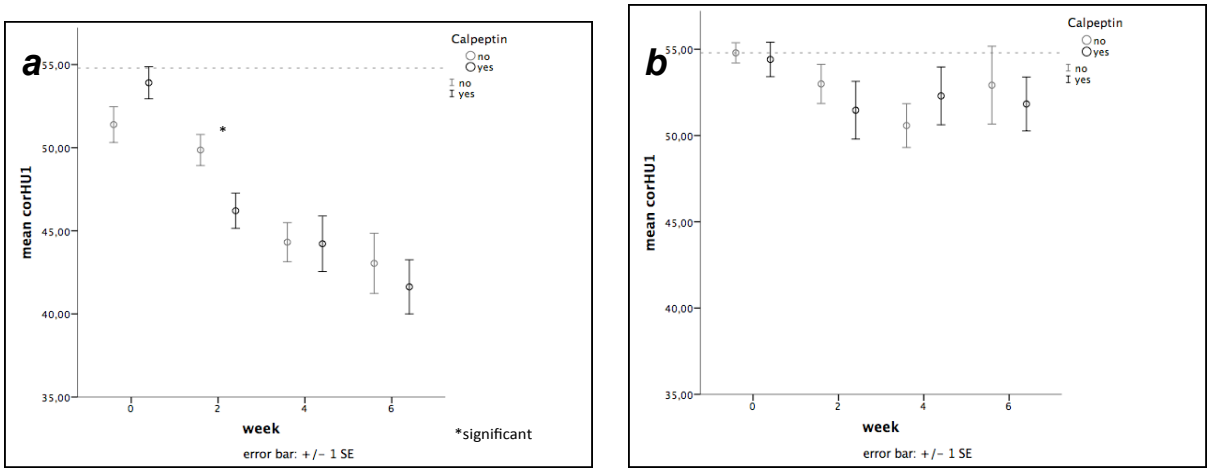

Figure 11. Mean coronal density over time measured in CT. As reference (54.79 ± 0.59) of the original density served the mean muscle density of the control group on the CLAT side in week 0, represented here by the grey dashed line. **(a)** OP side: Constant reduction of the density over the six weeks. Overall there are no significant differences between the CALP and the control group except in week 2, where the CALP group shows a lower density. **(b)** CLAT side: Constant muscle density. Overall there are no significant differences between the CALP and the control group.

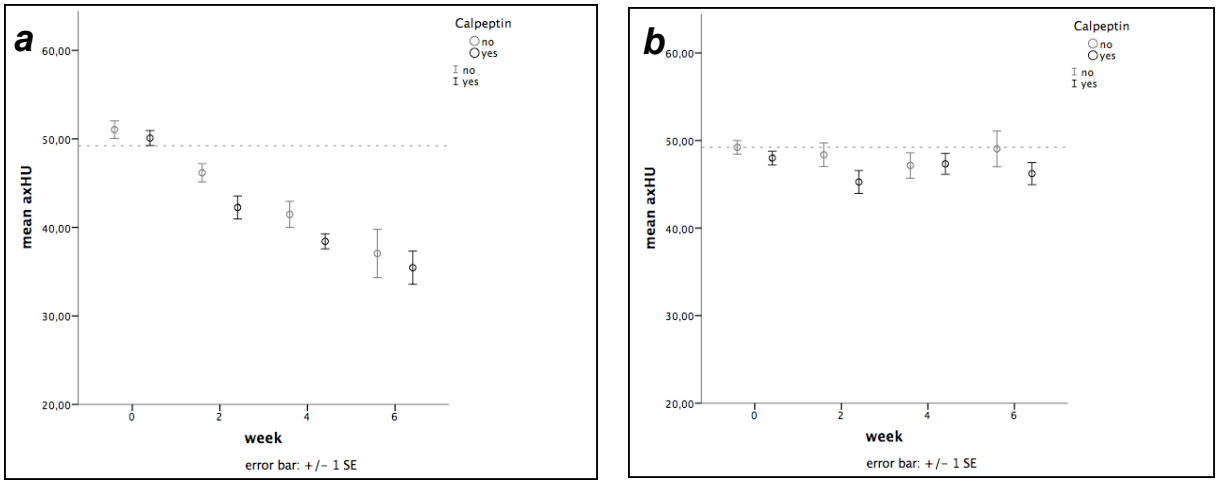

Figure 12. Mean transverse muscle density over time measured in CT. As reference (49.22 ± 0.78) of the original density served the mean muscle density of the control group on the CLAT side in week 0, represented here by the grey dashed line. **(a)** OP side: In both groups the density decreases over time. There are no significant differences between the groups. **(b)** CLAT side: Mainly constant muscle density. Overall there are no significant differences between the CALP and the control group.

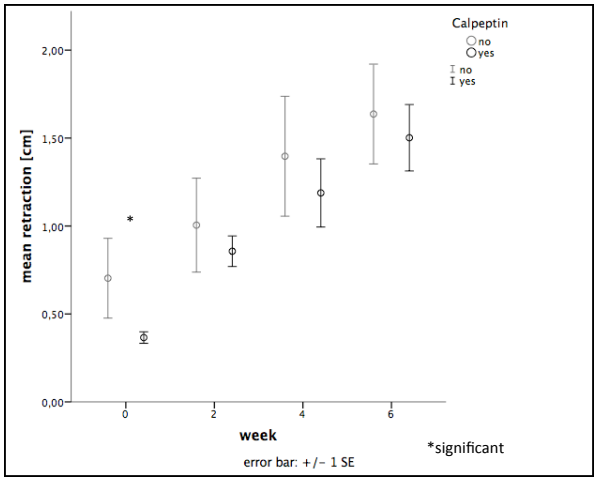

Figure 13. Mean fracture fragment retraction over time measured in CT. Constant increase of retraction over the six weeks. There are significant differences between the CALP and the control group in week 0.

Table 2		week 0			week 2			week 4			week 6		
OP	Reference	CALP	control	P	CALP	control	P	CALP	control	P	CALP	control	P
Muscle length [cm]	18.75 ± 0.22	16.38 ± 0.17	15.51 ± 0.39	**0.028**	15.58 ± 0.12	14.87 ± 0.44	0.177	15.35 ± 0.16	14.45 ± 0.31	**0.011**	14.87 ± 0.13	14.20 ± 0.42	0.179
CSA [cm^2]	15.20 ± 0.66	14.61 ± 0.62	15.30 ± 0.75	0.509	15.40 ± 0.52	14.59 ± 0.88	0.409	14.91 ± 0.54	15.27 ± 1.06	0.744	13.54 ± 0.62	13.44 ± 0.79	0.923
Fatty muscle infiltration [HU]													
• coronal	54.79 ± 0.59	53.91 ± 0.96	51.39 ± 1.08	0.128	46.21 ± 1.06	49.86 ± 0.93	**0.042**	44.22 ± 1.67	44.32 ± 1.17	0.969	41.63 ± 1.63	43.04 ± 1.81	0.593
• transverse	49.22 ± 0.78	50.10 ± 0.85	51.04 ± 1.00	0.508	42.25 ± 1.30	46.18 ± 1.03	0.067	38.43 ± 0.85	41.47 ± 1.48	0.073	35.45 ± 1.87	37.06 ± 2.73	0.626
Retraction [cm]	0.70 ± 0.23	0.37 ± 0.03	0.70 ± 0.23	**0.018**	0.86 ± 0.09	1.01 ± 0.27	0.820	1.19 ± 0.19	1.40 ± 0.34	0.591	1.50 ± 0.19	1.64 ± 0.28	0.591
CLAT	Reference	CALP	control	P	CALP	control	P	CALP	control	P	CALP	control	P
Muscle length [cm]	18.75 ± 0.22	19.19 ± 0.19	18.75 ± 0.22	0.187	19.16 ± 0.25	18.53 ± 0.22	0.130	19.13 ± 0.27	18.42 ± 0.28	0.111	19.10 ± 0.29	18.68 ± 0.36	0.387
CSA [cm^2]	15.20 ± 0.66	14.40 ± 0.52	15.20 ± 0.66	0.375	14.46 ± 0.51	15.06 ± 0.75	0.291	14.78 ± 0.45	15.40 ± 0.98	0.519	14.85 ± 0.60	15.37 ± 1.03	0.646
Fatty muscle infiltration [HU]													
• coronal	54.79 ± 0.59	54.41 ± 1.00	54.79 ± 0.59	0.820	51.47 ± 1.67	52.99 ± 1.14	0.555	52.30 ± 1.67	50.58 ± 1.27	0.498	51.83 ± 1.56	52.92 ± 2.26	0.437
• transverse	49.22 ± 0.78	48.00 ± 0.79	49.22 ± 0.78	0.344	45.27 ± 1.30	48.37 ± 1.34	0.156	47.33 ± 1.19	47.15 ± 1.45	0.927	46.21 ± 1.26	49.05 ± 2.03	0.216

Table 2. Effect of calpeptin on different parameters over time. Data refer to mean ± standard error, *p*-values for the effects of calpeptin on different muscle related parameters based on an unpaired t test.

6. Discussion

6.1. Main Results

Muscle length decreases over the time in both groups, whereas only in the early phase a difference in favour of the CALP group can be shown. The muscle seems to be paralysed by the calpain inhibitor and therefore shows a weaker contraction as the control group.

The CSA decreases drastically beyond four weeks of tendon release in both groups. This supports the assumption that muscle atrophy has occurred.

Decrease of densities in the coronal as well as in the transverse plane can already be observed early, whereas no difference appears between the two groups. This means fatty infiltration has taken place in both groups independently of the medication.

The retraction increases over time in both groups. Since CALP reduces the muscle strength, the muscle retracts less in the CALP group compared to the control group. Nonetheless differences between the two groups appear only in the early stages.

The CLAT side, which served as control, doesn't show any irregularities and all parameters remain mostly constant as expected, given that no interventions were performed.

6.2. Strengths and Shortcomings

The measurements of the fatty muscle infiltration were made only in one slice of the muscle and with the assumption that the Hounsfield Units reflect the fatty infiltration.

It was not histologically validated.

As well the measures of muscle CSA may underestimate the loss in muscle material, which can occur through a reduced length of muscle.

6.3. Implications

The results show that the muscle retracts very quickly and this has probably an association with calpain. Since mechanical factors regulate the muscle mass, there is a possibility of calpeptin, by sustaining the muscle length, preventing degenerative process.

Given that the retraction of the muscle is a problem for muscle reconstruction, the immediate application of calpeptin expands the time span for successful surgical procedure.

This is especially relevant given that the prevalence of rotator cuff tears in human is 20.7% increasing with age [18].

6.4. Unanswered Questions

Our results do not expose the mechanism underlying the reduced muscle retraction by calpeptin.

Possibly different mechanisms contribute to the early and late effects of calpeptin, such as reduced contractility and loss of muscle material (atrophy).

6.5. Conclusion

With calpeptin a positive effect on muscle length and retraction can be shown mainly in the first hour after tendon release.

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8. Curriculum vitae

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9. Decleration

Masterarbeit

Ich erkläre ausdrücklich, dass es sich bei der von mir im Rahmen des Studiengangs Humanmedizin eingereichten schriftlichen Arbeit mit dem Titel

"The Effect of Calpain-Inhibitor on Muscular Degeneration after Rotator Cuff Tear: A Randomized Clinical Trial in Sheep"

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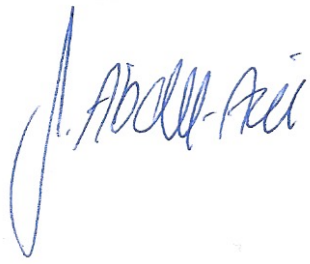
Unterschrift: 

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